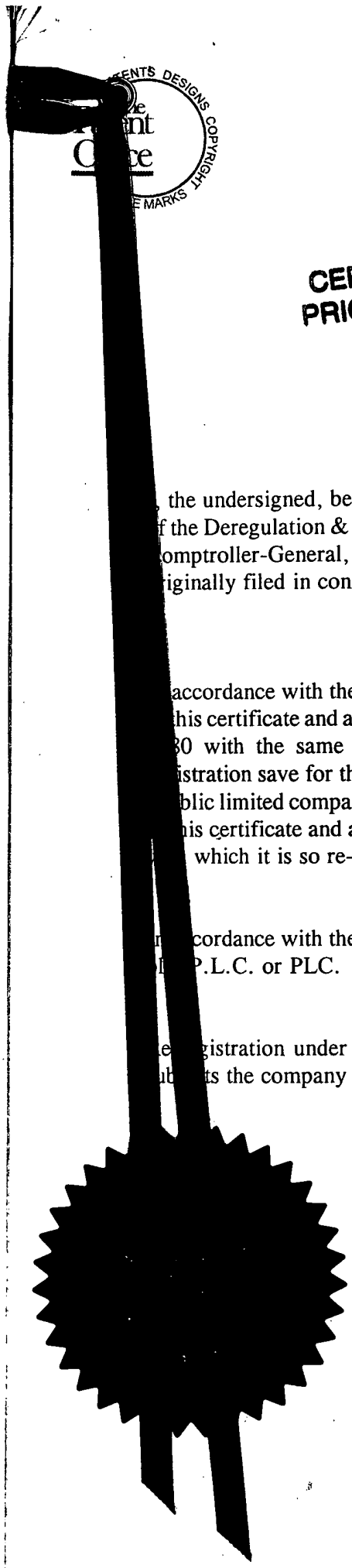

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Selection Method

The invention relates to a selection system which permits the selection of polypeptides displayed in a phage display system.

Viruses have been used for the display of peptides and proteins [Bass, S., R. Greene, and J.A. Wells. (1990). Hormone Phage: An Enrichment Method for Variant Proteins With Altered Binding Propertics. *Proteins*. **8**, 309-314, Smith, G.P. (1985). Filamentous Fusion Phage: Novel Expression Vectors That Display Cloned Antigens on the Virion Surface. *Science*. **228**, 1315-1317, McCafferty, J., *et al.* (1990). Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*. **348**, 552-554]. In particular filamentous bacteriophage have been used for display of proteins and peptides by fusion of the genes encoding the proteins or peptides to the gene encoding a phage coat protein. As the fusion gene is encapsidated in the phage that is displaying the fusion protein, this provides a linkage of phenotype and genotype. Repertoires of proteins can be encoded by a population of phage, and the rare phage encoding proteins with predefined binding activities isolated by binding to solid phase. In this way synthetic human antibodies of predefined antigen-binding specificity have been selected from repertoires of antibody fragments assembled from different structural elements [Winter, G., *et al.* (1994). Making Antibodies by Phage Display Technology. *Annual Review of Immunology*. **12**, 433-455]. As the antibody needs to be folded to bind antigen, selection for binding also selects for folding. This principle has also been used for selection of folded peptides where binding is mediated by a discontinuous epitope [Riddle, D.S., *et al.* (1997). Functional rapidly folding proteins from simplified amino acid sequences. *Nature Structural Biology*. **4**(10), 805-809; Braisted, A.C. and J.A. Wells. (1996). Minimizing a binding domain from protein A. *Proc. Natl. Acad. Sci. USA*. **93**, 5688-5692; O'Neil, K.T., *et al.* (1995). Thermodynamic Genetics of the Folding of the B1 Immunoglobulin-Binding Domain From Streptococcal Protein G. *Proteins: Structure, Function, and Genetics*. **21**, 11-21; Gu, H., *et al.* (1995). A phage display system for studying the sequence determinants of protein folding. *Protein Science*. **4**, 1108-1117].

However, all of the systems in current use rely on a binding activity in the polypeptide to be selected in order to perform the isolation of the desired display bodies from those which do not encode polypeptides having a desired binding activity. This places a limitation on available display systems to the selection of folded polypeptides which possess a binding activity. It would be desirable to have a means for selection of displayed proteins or polypeptides that is independent of the binding activity thereof.

Proteolytic cleavage requires the polypeptide chain to bind and adapt to the specific stereo chemistry of the protease active site, and therefore to be flexible, accessible and capable of local unfolding [Hubbard *et al.* 1994, Protein Science 3, 757-768; Fontana *et al.* 1997, Folding & Design 2, R17-R26]. This results in folded proteins often being resistant to proteolysis and unfolded proteins being sensitive thereto.

Although attempts have been made to screen for folded proteins by their ability to survive degrading enzymes in bacteria [Kamtekar *et al.* 1993, Science 262, 1680-1685; Davidson *et al.* 1994, PNAS 91, 2146-2150; Davidson *et al.* 1995, Nature Struct. Biol. 2, 856-864], such methods do not allow for selection if bacterial growth or survival does not depend on the function of the folded protein. Thus, these systems are only applicable to a small minority of polypeptides which one might wish to select according to the ability to fold.

It has previously been shown that the insertion of a peptide sequence between a proteolytically stable tag fused to the minor phage coat protein p3 and the p3 protein itself, followed by proteolysis, provides a means to select for phages bearing peptide sequences that are susceptible to proteolysis [Matthews & Wells 1993, Science 260, 1113-1117]. In these experiments, phage are bound to an affinity resin binding an N-terminal, proteolytically stable tag on the phage. If the bound phage are subjected to proteolysis and elution, only phage with cleavable sequences are eluted. This method is used to identify, among a repertoire displayed on phage, amino acid sequences suitable as substrates for proteases. The sequences introduced are short and would not be

capable of folding independently. Moreover, the system selects specifically for eluted rather than bound phage.

Summary of the Invention

The present invention concerns the introduction of polypeptides comprising protease cleavage sites into the sequences of the viral coat protein, and the exposure of the virus to a protease such that said protease cleavage sites are cleaved.

Accordingly, in a first aspect, the invention provides a method for selecting a virus, comprising the steps of:

(a) introducing a polypeptide comprising a cleavable site positioned between a stable tag fused to the N-terminus of a viral coat protein and the coat protein;

(b) binding the virus to a ligand via the tag;

(c) subjecting the virus to cleavage with a cleaving agent capable of cleaving the cleavable site unless protected by folding in the polypeptide;

(d) eluting the phage thereby cleaved from the bound tag; and

(e) recovering the virus which remain bound to the ligand.

Steps (c) and (b) may be reversed, such that virus is subjected to cleavage before binding to the ligand.

Moreover, the invention provides the use of a virus encoding a polypeptide positioned between a stable tag fused to the N-terminus of a viral coat protein and the coat protein for the selection of polypeptides capable of folding.

Brief Description of the Figure

Figure 1 shows the fd vectors fd-1, fd-2 and fd-3. The gene for the H102A mutant of Barnase is introduced by subcloning into fd-DOG [Hoogenboom *et al.*, 1991 NAR 19:4133-4137] after PCR amplification with suitable oligonucleotides using the

restriction sites ApaL1 (at the Barnase 5' end) and Not1 to create fd-3. Sequences indicated in fd-1 and fd-2 are created after cloning pairs of suitable, kinased oligonucleotides into the Pst1 site of fd-3.

Detailed Description of the Invention

Definitions

Virus As used herein, "virus" refers to an infective inocculum of virions, which may incorporate cleavage sites, optionally as part of heterologous polypeptides encoded by the viral genome. Thus, "virus" may refer to a plurality of virions, such that it may encode a repertoire of polypeptides; alternatively, as the context requires, it may be used to denote a single virion. The term "virus" includes any suitable virus which may incorporate a cleavage site, either naturally or through manipulation. A preferred virus for use in the present invention is bacteriophage, preferably filamentous bacteriophage.

Polypeptide The term "polypeptide" is used generally to denote polypeptides which are incorporated as fusions to viral coat proteins. Polypeptide is used interchangeably with "protein" herein and denotes a polypeptide which is displayed by the virus for selection purposes. Displayed polypeptides encoded in the virus of the invention as N-terminal fusions with virion polypeptides are preferably heterologous polypeptides which are potentially capable of folding to a folded conformation whilst incorporated in the virion polypeptides. Substantially any polypeptide capable of folding may be selected for by the method of the present invention, including structural polypeptides, polypeptides having enzymatic activity and polypeptides having binding activity, including antibodies and antibody fragments. Cleavable sites may preferably be present in the polypeptides, and may be naturally-occurring or may be engineered into the polypeptide or into a linker peptide attached thereto.

Cleavable site A site capable of cleavage when exposed to a cleaving agent. In the present invention, the use of protease cleavage sites, capable of being cleaved with

proteases, is preferred. Protease cleavage sites may be part of, or incorporated in, polypeptides according to the invention; alternatively, it may be independently engineered into the coat protein of the virus. A feature of the cleavable site is that it should either be absent from the virus other than at the site of its specific insertion according to the present invention, or otherwise inaccessible to cleavage, or present only in viral proteins which are not required after virion assembly to mediate infection.

Repertoire A repertoire is a collection of members, preferably polypeptides, which differ slightly from each other in a random or partially randomised manner. Preferably, a repertoire of polypeptides is a collection of variant polypeptides which preferably incorporate random or partially randomised mutations. As used herein, a repertoire preferably consist of 10^4 members of more. A repertoire advantageously comprises a very large number of members, typically between 10^8 and 10^{11}, and potentially 10^{14} or higher. From a repertoire, the invention allows the selection of members which have the ability to fold, preferably into an active conformation.

Tag A tag is any suitable entity capable of binding to a *ligand* which may be used to isolate a virus by the method of the present invention. Accordingly, the tag is resistant to the cleaving agent used in the method of the invention. Examples of tag/ligand pairs include barnase/barstar, avidin/biotin, antibody or antibody fragments and ligands, chelating groups and chelates, for example metals, and the like.

Description of Preferred Embodiments

The insertion of a polypeptide between the stable tag fused to the N-terminus of the viral coat protein and the coat protein itself, followed by cleavage, provides a means of selection for virus bearing proteins that are resistant to proteolysis and are folded. Thus only virions, whose inserted polypeptide is not degraded, will keep the tag fusion as part of their coat, and only these virions can therefore be captured by affinity purification using this tag. After elution the affinity captured phases from the ligand,

these phages can be propagated and subjected to further rounds of the same selection procedure.

Alternatively, virions may be bound to an affinity matrix, comprising a ligand for the tag, prior to cleavage. The cleaving agent may subscquently be added, and only resistant phage will be retained on the matrix. These may then be eluted as required.

Suitable matrices include columns, beads and other surfaces to which a ligand for the tag is bound.

According to the present invention, reference to selection may be interpreted as a reference to screening, since the same processes may be used to screen phage, as will be apparent to persons skilled in the art.

Cleavable sites may be naturally part of the polypeptide, but preferably they are engineered therein. Preferred cleavable sites include protease cleavage sites, which may be found in polypeptides or engineered as an integral part of their sequence. Typically, protease cleavage sites may be defined in terms of amino acid sequences which are susceptible to cleavage by a protease. For example, the invention encompasses the use of protease cleavage sites cleavable by one or more of the proteases trypsin (cleaves at Lys, Arg), chymotrypsin (Phe, Trp, Tyr, Leu), thermolysin (small aliphatic residues), subtilisin (small aliphatic residues), Glu-C (Glu), Factor Xa (Ile/Leu-Glu-Gly-Arg), Arg-C (Arg) and thrombin.

In a preferred embodiment, the virus for use in the present invention is a bacteriophage, preferably filamentous bacteriophage. Filamentous bacteriophage is widely used in phage display techniques for the selection of polypeptides from phage libraries encoding a large repertoire thereof. Conventionally, the repertoire of polypeptides is inserted in the p3 protein of filamentous bacteriophage, but any other suitable coat protein may be employed within the scope of the present invention. In the present invention, the polypeptide is advantageously fused to the N-terminus of p3.

The protcase cleavage sites are preferably incorporated into heterologous polypeptides, for example those polypeptides encoded in the form of a repertoire in a phage library. As folded polypeptides or proteins are often resistant to proteolysis and unfolded proteins are sensitive, cleavage requires the polypeptide chain to bind and adapt to the specific stereochemistry of the protease active site, and therefore to be flexible, accessible and capable of local unfolding [Hubbard, S.J., F. Eisenmenger, and J.M. Thornton. (1994). Modeling studies of the change in conformation required for cleavage of limited proteolytic sites. *Protein Science*. 3, 757-768; Fontana, A., *et al.* (1997). Probing the partly folded states of proteins by limited proteolysis. *Folding & Design*. 2, R17-R26].

The possible selection of polypeptides from a repertoire which, through variation or mutation, do not contain a recognition sequence for any particular protease used in this method, can be circumvented in two ways. For example, the use of a cocktail of proteases with very distinct recognition sequences would ensure that all polypeptides should be cleavable, if not protected by their folded status. Alternatively, a phage repertoire of polypeptides to bc selected could be partially denatured, such that the inserted polypeptide unfolds but the phage and the N-terminal tag remains intact. Proteolytic digestion followed by affinity purification would remove all phages form the repertoire, which have escaped proteolysis due to the lack of protease recognition sequences in the polypeptide. Phages not bound by the resin, contain only phages, which contain the protease recognition sequence in the polypeptide displayed and which may or may not escape proteolysis under non-denaturing conditions. Thus these would be subjected to proteolytic selection based on protection by the folding status of the polypeptide displayed.

The selection process may also be used for the identification of interacting protein elements. Thus if two such elements linked by a polypeptide comprising protease cleavage sites were cloned between the N-terminal, proteolytically stable tag for display on phage, the only phages after proteolysis, that can be captured via affinity

binding to the tag, should be those in which the tag and the p3 protein are held together by non-covalent interactions between the interacting protein elements.

The invention is further described in the following examples, for the purposes of illustration only.

Example 1. Resistance to proteolysis of an N-terminal tag in a phage coat protein.

The enzymatically non-active mutant H102A of the bacterial RNase Barnase [Meiering *et al.* 1992, JMB 225, 585-589], which comprises a highly stable single domain protein of 110 residues, is fused (Fig. 1) to the N-terminus of the minor phage coat protein p3 followed by either the amino acid sequence (phage fd-1: LQTPSGPAGGAAA) or the peptide sequence (phage fd-2: LQAKSRSAGGAAA) before the first residue of the p3 protein in phage fd-TET (Zacher *et al.* 1980, Gene 9, 127-140]. Both phages but not fd-TET can be captured with high efficiency on a Streptavidin surface using the biotinylated C40A,C82A double mutant Barnase inhibitor Barstar [Hartley 1993, Biochemistry 32, 5978-5984]. Specific capture of phages I and 2 is demonstrated through elution at pH 2 and titration of $6x10^5$ infectious particles as compared with elution of $2x10^2$ non-tagged phage fd-TET from a volume of 100 µl in a Streptavidin coated microtitre well. In all cases $1x10^7$ infectious particles are incubated in the well for binding. Phage binding is further demonstrated through detection of bound phage with an anti-M13 phage antibody - horse radish peroxidase (HRP) conjugate and a suitable peroxidase substrate.

Proteolytic treatment of phage fd-1 with both Trypsin or Thermolysin in TBS-Ca buffer (25 mM Tris, 137 mM Nacl, lmM $CaCl_2$, pH 7.4) at concentrations up to at least 1 ng/µl before capturing in the Streptavidin well coated with biotinylated Barstar does not result in loss of binding as detectable by the anti-Ml3 antibody-HRP assay. Thus, the protein-tag Barnase, the peptide sequence between Barstar and the p3 coat protein and the p3 coat protein itself in phage fd-1 are, as expected, resistant to both tryptic and thermolytic cleavage. Barnase and the p3 protein are resistant although they contain

numerous basic (K, R) and aromatic (F, Y, W) residues, which serve as preferred substrate residues for Trypsin and Thermolysin respectively. Both proteins are protected from cleavage due to their stably folded tertiary structure. The peptide sequence between Barnase and p3 in phage is protected from cleavage, because it does not contain any basic or aromatic residues and therefore no high affinity substrate for the two proteases used.

Example 2. Non-resistance to proteolysis of phages with Barnase-p3 fusions containing a peptide sequence with Trypsin substrate residues.

Proteolytic treatment of phage fd-2 (see Example 1) with Trypsin at a concentration of 1 ng/µl before capturing in a Streptavidin well coated with biotinylated Barstar results in complete loss of binding as detectable by the anti-M13 antibody-HRP assay. Non-resistance to tryptic cleavage of the peptide sequence inserted between Barnase and p3 is expected, because it contains the basic residues K and R, which provide suitable recognition sites for Trypsin, and because the short, randomly chosen peptide sequence is not expected to fold into a stable structure.

The fusion of Barnase as an N-terminal tag for capture followed by a polypeptide sequence (to be selected for folding), with the minor phage coat protein p3 and exposure of this phage to proteases therefore represents a suitable design to select for proteolytic resistance of a polypeptide inserted between Barnase and p3.

Example 3. A vector for the cloning of peptide encoding DNA fragments for display in Barnase-p3 fusions.

For the cloning of (poly)-peptide encoding DNA fragments and their display for selection between Barnase and p3, the phage fd-3 is constructed (Fig. 1). Phage fd-3 comprises the H102A mutant of Barnase N-terminally fused to the p3 gene of phage fd-TET. Between the codon for the last residue of Barnase and the first residue of p3 is the nucleotide sequence CTG CAG GCG GTG CGG CCG CA. This sequence contains

a PstI DNA restriction site for insertion of DNA fragments flanked by PstI restriction sites. The, sequence further introduces a frame shift between Barnase and p3, which prevents expression of the correct p3 reading frame in fd-3. Phage particles of pha fd-3 do not contain the protein p3 and are non-infectious.

Phage fd-3 is therefore well suited as a cloning vector as vectors without PstI DNA inserts after ligation are not propagated during selection. Statistically 1 out of 3 random DNA inserts in the PstI restriction site will create an open reading frame spanning Barnase, the insert itself and p3 and result in infectious phage particles containing p3 in the phage coat. In these recombinant clones Barnase is followed by the insert, which is then followed by the amino acid residues AGGAAA before the start of the p3 protein. This AGGAAA should provide enough flexibility in the fusion protein to enable the infectivity function of p3 and the access of the protease to the N-terminal appendices of p3.

Example 4. The cloning of random DNA fragment dimers from the *E.coli* genome into fd-3.

Genomic DNA from the *E.coli* strain TGI is amplified in 30 cycles of a polymerase chain reaction (PCR) with an annealing temperature of 48°C using the oligonucleotide SN6MX (5'GAG CCT GCA GAG CTC AGG NNN NNN-3'), which comprises 6 degenerate positions at the extendible 3' end to ensure random priming. In a second step of 30 PCR cycles with an annealing temperature of 52°C primary PCR products are extended by reamplification with the oligonucleotide XTND (5'-CGT GCG AGC CTG CAG AGC TCA GG-3'). Products with a length of around 150 bp from this reaction are purified from an agarose gel and reamplified in 30 PCR cycles using an annealing temperature of 52°C and the oligonucleotide XTND. These reamplified 150 bp fragments are partially digested with SacI and ligated for dimerisation. Ligated products are reamplified in a further 10 PCR cycles with an annealing temperature of 44°C followed by a 30 PCR cycles with an annealing temperature of 55°C using the oligonucleotide XTND. The annealing temperatures are

chosen to discriminate against priming of the oligonucleotide in the middle of the dimerised fragments. The reaction product is size purified twice on an agarose gel to remove monomers and oligomers (non-dimers).

The final dimer fraction is amplified by PCR using an annealing temperature of 55°C and the oligonucleotide XTND on a large scale, digested with PstI (site indicated in italics in oligonucleotides) and ligated into the PstI digested and phosphatased vector fd-3 (see Example 3). After electroporation into *E.coli* AB2300 [Eggertsson 1968, Genet. Res.11, p.15] a repertoire of $3.6x10^7$ recombinants is obtained. After analysis of the percentage of clones harbouring a dimer-insert, statistically $3.5x10^6$ recombinants contain in-frame inserts without an TGA stop codon leading to premature termination of the p3 fusion protein and thereby the production of non-infectious phage. The other two stop-codons of the genetic code (TAA, TAG) are partially suppressed in the *E.coli* strain AB2300. A functionality of all three stop-codons would reduce the repertoire size to $8.5x10^5$ recombinants with no-stop, in-frame inserts, which lead to the production of infectious particles.

Reinfection of AB2300 *E.coli* cells with phage produced from the initial population of transformed cells yields, according to sequence analysis of twenty randomly picked clones, a library of infectious phages containing almost exclusively Barnase-(in-frame, no-stop-dimer-insert)-p3 fusions. Non-infectious phages arising from vector without insert and from vector with out-of-frame or stop-codon containing inserts will not have been propagated in the infection step. The vector fd-3 has therefore been shown to be suitable to create a repertoire of polypeptides randomly generated through dimerisation of DNA fragments from the *E-coli* genome.

Example 5. Proteolytic stability of polypeptides displayed on phage as fusions inserts in Barnase-p3.

The repertoire of $3.5x10^6$ polypeptides displayed as an inserted fusion between Barnase and p3 on phage fd (see Example 4) is subjected to proteolytic digestion with

Trypsin and Thermolysin alone and in a mixture of the two (1 ng/µl each). After proteolysis with Trypsin 0.01 % of the phage could be captured with biotinylated Barstar bound to a Streptavidin coated microtitre well plate. After proteolysis wi Thermolysin and a mixture of Trypsin and Thermolysin 0.005% of the phage are captured with biotinylated Barstar bound to a Streptavidin coated microtitre well plate.

When phage propagated from the eluted (and presumably at least partially proteolytically protected) phage is again selected by proteolysis and affinity capture, about 0.1% (i.e. ten and twenty times more phage than initially) is captured after the selection. This result indicates that the method is able to enrich for proteolytically protected phage.

Claims

1. A method for selecting a virus, comprising the steps of:

(a) introducing a polypeptide comprising a cleavable site positioned between a stable tag fused to the N-terminus of a viral coat protein and the coat protein;

(b) binding the virus to a ligand via the tag;

(c) subjecting the virus to cleavage with a cleaving agent capable of cleaving the cleavable site unless protected by folding in the polypeptide;

(d) eluting the virus thereby cleaved from the bound tag; and

(e) recovering the virus which remain bound to the ligand.

2. A method according to claim 1, wherein steps (b) and (c) are performed in reverse order.

3. A method according to claim 1 or claim 2, wherein the virus encodes a repertoire of polypeptides.

4. A method according to claim 3, wherein a plurality of cleaving agents are used in order to ensure cleavage of all polypeptides wherein the cleavable site is not protected by folding.

5. A method according to claim 3, wherein the virions encoding polypeptides not susceptible to cleaving with the cleaving agent are removed prior to selection.

6. A method according to any preceding claim, wherein the virus is bacteriophage.

7. A method according to claim 6, wherein the bacteriophage is filamentous bacteriophage.

8. A method according to claim 7, wherein the coat protein is the minor phage coat protein 3 of filamentous bacteriophage.

9. A method according to any one of claims 3 to 8, wherein the nucleic acid encapsidated by the virus encodes a repertoire of sequences.

10. A method according to any preceding claim, wherein virions that are resistant to cleavage are propagated by infection.

Abstract

The invention provides a method for selecting a virus, comprising the steps of:

(a) introducing a polypeptide comprising a cleavable site positioned between a stable tag fused to the N-terminus of a viral coat protein and the coat protein;

(b) binding the virus to a ligand via the tag;

(c) subjecting the virus to cleavage with a cleaving agent capable of cleaving the cleavable site unless protected by folding in the polypeptide;

(d) eluting the virus thereby cleaved from the bound tag; and

(e) recovering the virus which remain bound to the ligand.

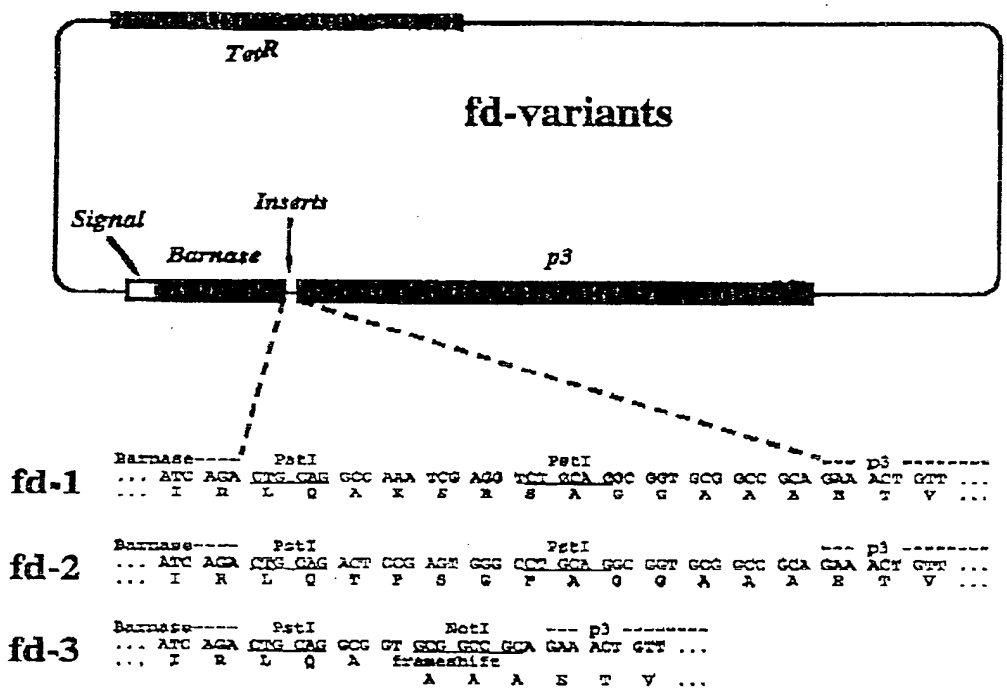

Figure 1. The fd vectors fd-1, fd-2 and fd-3. The gene for the H102A mutant of Barnase was introduced by subcloning into fd-DOG [Hoogenboom et al. 1991, NAR 19, 4133-4137] after PCR amplification with suitable oligonucleotides using the restriction sites ApaLI (at the Barnase 5' end) and NotI to create fd-3. Sequences indicated in fd-1 and fd-2 were created after cloning of pairs of suitable, kinased oligonucleotides into the PstI site of fd-3.

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